A Study of the Initial Velocity in the Hydrolysis of Sucrose by Invertase.

DISSERTATION

A Study of the Initial Velocity in the Hydrolysis of Sucrose by Invertase.

DISSERTATION

Submitted in Partial Fulfillment of the requirements
for the Degree of Doctor of Philosophy in
the Faculty of Pure Science of
Columbia University.

BY

HAROLD LESTER SIMONS, A.B., A.M.

NEW YORK CITY

1921

TO MY
FATHER AND MOTHER

ACKNOWLEDGMENT

The author desires to express his gratitude to Professor J. M. Nelson, at whose suggestion this work was carried out. To his fruitful suggestions and to his kindly encouragement, this research owes whatever merit it may possess.

ABSTRACT OF DISSERTATION

1. What was attempted?

2. In how far were the attempts successful?

3. What contribution actually new to the science of Chemistry has been made?

1. In view of the fact that many investigators have extrapolated the initial portion of the curve representing the hydrolysis of sucrose by invertase by assuming that the function was initially linear and due to the importance of knowing the actual course of the inversion at its start, an attempt has been made to determine:

(a) Whether this condition actually exists;

(b) In the case of its actual existence, whether it is due to lack of sufficiently great experimental precision or is due to some peculiarity in the reaction.

2. It has been found that

(a) In general, the initial course of the curve representing the hydrolysis is linear;

(b) Under certain conditions, however, the velocity actually rises to a maximum;

(c) The observed linear part of the curve is due mainly to a peculiarity of the reaction.

3. It has been shown that

(a) By actual measurement the velocity of the inversion of sucrose by invertase is, in general, constant at the start. Thsi is the usual case even in the presence of added substances. However, under certain conditions, the velocity has been observed to go through a maximum before the gradual decrease sets in;

(b) This constant or increasing velocity is only evident at the beginning of the hydrolysis;

(c) These observations—particularly in view of the fact that the initial velocity at times increases—strongly indicate that invertase action involves a series of consecutive reactions;

(d) Since the velocity often increases to a maximum during the earlier part of the reaction, the initial velocity cannot be taken as a true measure of the activity of the invertase. In the light of this fact, the theory of invertase action proposed by Colin and Chaudun (which depends upon the assumption that the rate of combination between sucrose and invertase is infinite) is untenable. This criticism likewise applies to the theory advanced by Michaelis and Menten which fails to account for some other facts disclosed by this investigation.

A STUDY OF THE INITIAL VELOCITY IN THE HYDROLYSIS OF SUCROSE BY INVERTASE

In determining the activity of hydrolytic enzymes, like invertase, the question arises as to what extent the velocity of the hydrolysis of the substrate can be taken as a measure of the activity or, in other words, how much of the enzyme present in the solution is in the active condition.

Henri[1] proposed

$$Kt = (1 + na)\ln\frac{a}{a-x} + (m - n)x \ldots\ldots\ldots (1)$$

as an equation for representing the kinetics for the hydrolysis of cane sugar by invertase. In his derivation of this equation (1), he like many others (Brown[2]) assumed one unit of invertase to combine (reversibly according to the Mass Law) with one mole of cane sugar and also with the inverted cane sugar as it was formed in the reaction. The terms *m* and *n* in the equation are the equilibrium constants of the cane sugar—invertase and inverted cane sugar—invertase compounds respectively. The K is the velocity coefficient of the hydrolysis, and a—x and x represent the concentrations of the cane sugar and inverted cane sugar at the time t. He also assumed that the velocity of combination between the enzyme and sugars is infinite when compared to the velocity of hydrolysis. Henri was unable to obtain experimental values for the mass law constants (*m* and *n*) and therefore resorted to a method based upon trial, assigning to *m* and *n* arbitrary values which gave fairly constant values for the velocity coefficient, K.

Hudson[3] objected to Henri's results on the ground that the latter had followed the course of the hydrolysis by permitting the reaction to take place in a polariscope tube and noticing the change in rotation of the sugar solution from time to time. The rotation values obtained in this way involve an error, since the α glucose and α fructose which are liberated from the cane sugar and undergo muta-rotation into their respective equilibrium mixtures of α and β isomers but slowly

under the acidity conditions which prevail in invertase reactions. When this error is avoided, then Hudson claims from his own results that the velocity of hydrolysis becomes directly proportional to the concentration of the cane sugar present, or in other words, that the reaction is mono-molecular and similar to acid hydrolysis. In spite of Hudson's results, which might belong to some special case, it has been quite firmly established that invert sugar, glucose and fructose exert a retarding influence on the velocity when cane sugar is hydrolyzed by invertase. (vide—Brown[2], Armstrong[4], and others.)

Michaelis and Menten's Method for Determining the Dissociation Constant of Sugar-invertase Compounds.

Michaelis and Menten[5], adopting Henri's theory concerning the mechanism of invertase action, proposed a method for determining experimentally the dissociation constants of the invertase-cane sugar and invertase-invert sugar compounds (i. e.—m and n in equation (1)) and thereby avoiding the difficulty encountered by Henri, whose equation contained three unknowns. According to them, at the beginning of the hydrolysis only cane sugar, cane sugar-invertase compound and water are present in the solution; and the velocity of hydrolysis is then assumed to be proportional to the concentration of the cane sugar-invertase compound. Since in this theory the Mass Law is considered to hold, by varying the initial concentration of the cane sugar and comparing the velocities corresponding to these different concentrations, they derive a relationship:

$$V = \frac{S}{S + m} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

which is similar in form to

$$(1 - \alpha) = \frac{H^+}{H^+ + k} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Equation (3) is the expression for the ionization of an acid in which the symbols have their customary significance.

NOTE—By plotting the concentration of the undissociated acid $(1 - \alpha)$ as ordinates and the logarithms of the concentration of the hydrogen ion, H^+, as abscissas, a curve is obtained from which the value of the dissociation constant can be obtained graphically.

In equation (2) S is the concentration of the free cane sugar and corresponds to H^+ in (3), V is proportional to the velocity of hydrolysis of the cane sugar and therefore is directly proportional to the concentration of the undissociated cane sugar-invertase compound and hence corresponds to $(1-\alpha)$ in (3). Therefore the dissociation constant, *m*, of the cane sugar-invertase combination can be obtained graphically just as the ionization constant, K, in equation (3) can be determined. As a consequence of this idea, *n* is resolved into two constant—one for glucose and the other for fructose, in combination with invertase. Each of these constants is determined by hydrolyzing the cane sugar in the presence of definite amounts of glucose or fructose, since the monose is considered to combine with a certain amount of the enzyme and thereby leaves less invertase to combine with and hydrolyze the cane sugar. If I represents the total enzyme present, P the amount combined with the cane sugar and T the amount combined with the glucose (or fructose, as the case may be), then the dissociation constant of the cane sugar invertase compound can be represented by

$$m = \frac{S \times (I - P - T)}{P} \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

and that for the glucose-invertase compound by

$$n = \frac{G + (I - P - T)'}{T} \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

in which equation S and G are the concentrations of free cane sugar and free glucose respectively. Since the velocity of hydrolysis is considered proportional to the concentration of the cane sugar-invertase compound, the relation between the initial velocities when glucose is present and when it is absent can be expressed by the equation

$$V : V_0 = P : P_0 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (6)$$

in which P and P_0 are the concentrations of the invertase-cane sugar compound when glucose is present and when it is absent. By combining (4), (5) and (6) the expression

$$n = \frac{Gm}{(S + m)\left(\frac{V}{V_o} - 1\right)} \quad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

is obtained, in which all the terms except n are known or capable of measurement. It was stated above that in measuring the relative velocities of hydrolysis of the cane sugar both in the absence and presence of invert sugar, glucose or fructose, Michaelis and Menten made use of the initial velocities in each case. They considered the velocity to be sufficiently constant at the beginning of the reaction so that the initial portion A in Figure III of the hydrolysis curve, obtained by plotting the change in rotation of the solution as ordinates and time as abscisas, would be practically a straight line and its slope with the time axis would be therefore the amount of cane sugar hydrolyzed divided by the time, or a measure of the velocity.

In Figure III is given the general shape of a curve for the hydrolysis of cane sugar when either invertase or acid is used as the catalyst. Acid hydrolysis of cane sugar obeys quite well the mono-molecular law,

$$k = \frac{1}{t} \log \frac{a}{a - x} \quad \ldots\ldots\ldots\ldots\ldots\ldots (8),$$

in which k is the velocity coefficient and a is the initial concentration of the cane sugar. When x, the amount of cane sugar hydrolyzed is plotted against the corresponding time, t, it will be found that the initial portion A of the curve is such in shape that it cannot be distinguished from a straight line by inspection, although from (8) it is known to be logarithmic. The general shape of the curve obtained when cane sugar is hydrolyzed by invertase is similar to that obtained when acid is used, the only difference being that in most cases when the velocity coefficients are calculated according to the mono-molecular law with invertase as the catalyst, it is found that they increase during the major portion of the reaction. This means that the invertase curve, in general, bends less towards the time axis than the acid curve.

Let us grant that is permissible to consider the portion of the hydrolysis curve corresponding to A in Figure III as the tangent to the curve at the beginning of the reaction and that it can be used as a measure of the initial velocity without introducing very much of an error. But Michaelis and Menten followed the same procedure when measuring retardation due

to invert sugar, glucose or fructose. Adding invert sugar, glucose or fructose—the orders of the magnitude of the retardation of glucose and fructose upon the reaction are the same—to the cane sugar solution before the hydrolysis by the enzyme has commenced is equivalent to moving the origin of the hydrolysis curve up into the B portion of the curve in Figure III because the composition of the sugar solution under these conditions would be the same as that of a cane sugar solution which had been undergoing hydrolysis for some time. If the products of the hydrolysis of the cane sugar by invertase have the same influence upon the reaction when added before the beginning of the reaction, it is evident that the first few experimentally determined points on the curve cannot lie on a straight line, because the curvature is so much more pronounced in the B portion than in the A. In other words, it is not possible (due to the shape of the B portion) for a curve drawn through the first few experimentally determined points to be sufficiently linear so that it can be regarded as tangent to the hydrolysis curve at the beginning when the origin of the curve lies in B—as is the case when glucose or invert sugar have been added previous to the start of the reaction. On the other hand, if the first few points do fall on a straight line or approximately straight line, as Michaelis and Menten claim, then it is necessary to conclude that the influence of the products of hydrolysis formed during the course of the hydrolysis is different from the influence they exert when added before the beginning of the reaction.

It was decided, therefore, to examine the initial velocity of the hydrolysis of cane sugar by invertase in the presence and absence of glucose and invert sugar at the beginning of the reaction. In Table I are recorded the results obtained. Samples were removed from the solution undergoing hydrolysis every five minutes during the first 25 minutes of the reaction. The enzyme action was interrupted in the removed samples by means of sodium carbonate, and the solutions examined in a 400 mm. tube in a polariscope. (For further details concerning the experimental procedure see the experimental part).

TABLE I.

Part A. 1 cc. Invertase solution per 100 cc. of solution. Temperature 25° Hydrogen ion conc. $10^{-4.4}$ moles per liter.

Conc. of Sucrose	Rotation after the reaction had continued for-minutes 0	5	10	15	20	25	∞
1.0%	2.62°	2.52°	2.42°	2.31°	2.195°	2.09°	—0.73°
1.5%	3.91	3.78	3.65	3.52	3.40	3.28	—1.08
2.0%	5.21	5.06	4.92	4.78	4.65	4.51	—1.45
3.0%	7.80	7.63	7.48	7.33	7.18	7.03	—2.24
4.0%	10.39	10.22	10.08	9.91	9.75	9.58	—2.96
5.0%	13.00	12.82	12.655	12.475	12.32	12.16	—3.71
6.0%	15.60	15.45	15.29	15.13	14.96	14.82	—4.51

Part B. Same solution as in A except 2 cc. of invertase solution per 100 cc.

Conc. of Sucrose	Rotation after the reaction had continued for-minutes 0	5	10	15	20	25	∞
1.0%	2.666°	2.445°	2.235°	2.033°	1.843°	1.658°	—0.655°
2.0%	5.258	4.980	4.705	4.433	4.175	3.924	—1.42
3.0%	7.858	7.539	7.240	6.936	6.645	6.357	—2.17
4.0%	10.450	10.124	9.807	9.479	9.172	8.867	—2.92
5.0%	13.078	12.748	12.439	12.116	11.799	11.500	—3.65
6.0%	15.641	15.303	14.995	14.673	14.371	14.073	—4.41

Part C. All solutions contain 1% conc. sugar at the start, but varying conc. of added glucose. Conc. of invertase, temperature and hydrogen ion conc. same as in B.

Conc. of Glucose	Rotation after the reaction had continued for-minutes 0	5	10	15	20	25	∞
0.0%	2.666°	2.445°	2.235°	2.033°	1.843°	1.658°	—0.655°
1.0%	4.693	4.519	4.346	4.171	4.012	3.855	1.381
3.0%	8.793	8.673	8.537	8.396	8.262	8.140	5.475
4.0%	10.863	10.765	10.635	10.503	10.392	10.285	7.55

NOTE—The figures above are the averages of several readings for each experiment and of two series of experiments. An estimation of the error would put it at slightly less than 0.01°. The last figures in B and C were retained as an average rather than because of its significance. Due to the addition of sodium carbonate solution and the presence of the optically active invertase, the above readings do not correspond exactly to the rotations which sugar solutions of these concentrations would have.

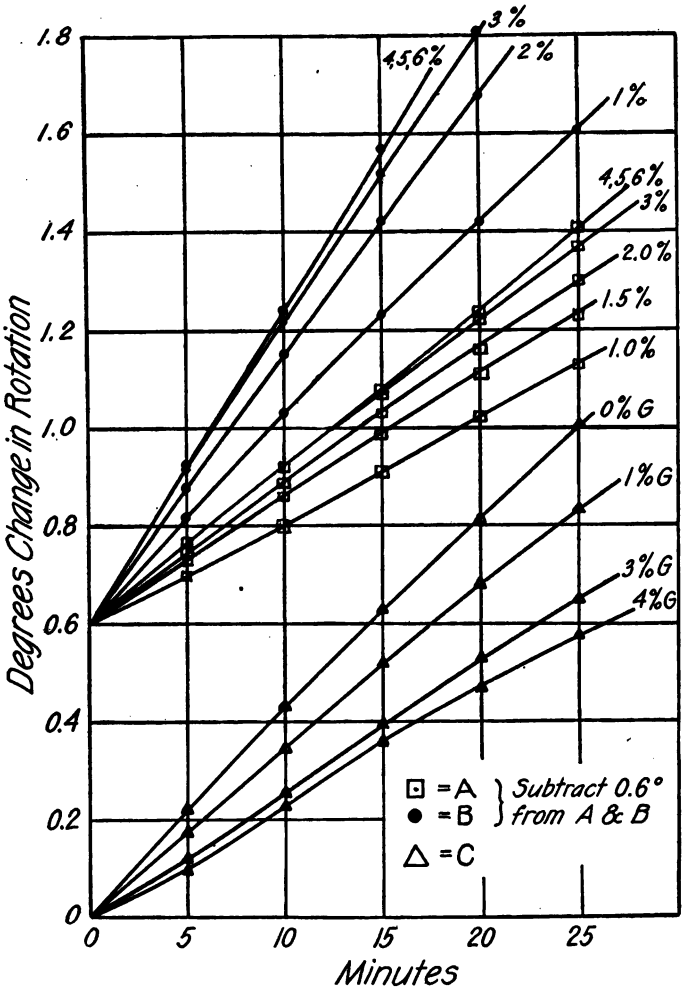

FIG. I

The data in Table I show that all these sugar solutions have practically constant *initial* velocities. The last two (Part C) containing 3% and 4% glucose appear to show a slight increase in velocity during the earlier periods of the reaction. This effect becomes more evident when the results are plotted as in Figure I. In the light of the above results, it therefore appears reasonable to regard the line passing through the first

few experimentally determined points on the hydrolysis curve as the tangent to the curve at its origin. In other words, Michaelis and Menten's method of determining the initial velocity seems to be all right. On the other hand, the fact that the initial velocity, when glucose is present in the cane sugar solution, is constant or increasing shows that a difference exists between it and the velocity after the initial period has been passed, as was suggested in the discussion of the difference between the A and B portions of the curve in Figure III. Consequently, there appears to be a time element involved, and the initial velocity is not comparable with the velocity prevailing after the hydrolysis has been going on for some time, even though in the two cases the compositions of the solutions may be the same. If this be true then the method of Michaelis and Menten, which depends upon the initial velocities, will not be applicable for measuring the actual velocities throughout the entire course of the reaction.

The Initial Velocity Involves a Time Element.

In order to gain more evidence concerning this time element which appears to exist between a reaction beginning and one that has been in progress for some time the following experiments were undertaken.

Solution A contained cane sugar and invert sugar (equal amounts of glucose and fructose) in such concentrations as to correspond to a 3% cane sugar solution that had undergone hydrolysis to the extent of about 42%. The initial velocity of hydrolysis of solution A was found to be constant just as had been observed in the experiments described in Table I, Part C, and the values are given in Table II, Part A.

TABLE II.

Hydrogen ion conc. $= 10^{-4.4}$.
Temp.=25°, 2.49 cc. invertase per 100 cc. of solution.

A		B
1.740 gm. Cane Sugar 0.6632 " Glucose 0.6632 " Fructose	per 100 cc. Sol.	3.0 grms. cane sugar per 100 cc. hydrolyzed to about 42%.

Time		Rotation	Time	Rotation
0.0	(61.50)	4.029°	59.99	4.120°
3.01	(64.51)	3.847	63.03	3.937
6.00	(67.50)	3.666	66.00	3.776
9.02	(70.52)	3.487	69.01	3.639
12.01	(73.51)	3.310	72.01	3.502
15.01	(76.51)	3.148	75.00	3.380
18.01	(79.57)	2.978	78.03	3.283

NOTE—These results are the mean of two series of experiments. The above values differ by no more than 0.005° from either of the individual experimental values.

For the sake of comparison, a 3% cane sugar solution B was permitted to undergo hydrolysis until it had reached about 42% inversion of the cane sugar. At this point the composition of B corresponded to approximately that of the initial concentration of A. The velocity of hydrolysis of solution B was determined after it had passed the 42% hydrolyzed stage and compared with the velocity of solution A. The values obtained are given under B in Table II.

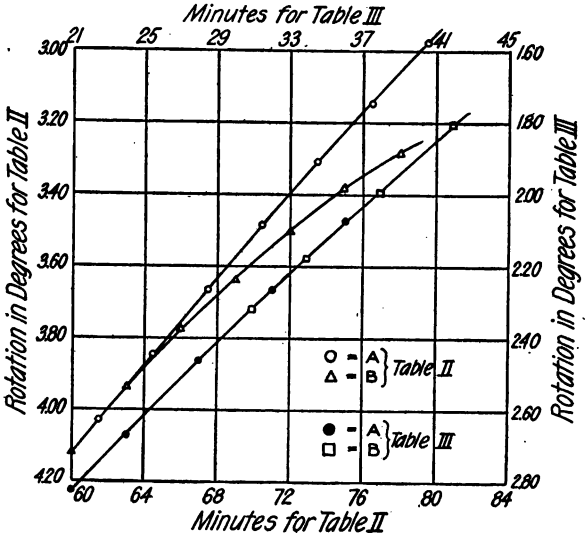

FIG. II

The difference between the velocities of solutions A and B is brought out more clearly by means of the curves in Figure

II. In order that these curves may be strictly comparable, it was necessary to adjust the time of solution A to that of solution B, since in this way both hydrolysis curves can be plotted together. The time for solution B, when its rotation was the same as the initial rotation of A (4.029°), was read off on the curve for solution B and found to be 61.50 minutes, and the values given in the parentheses under A in Table II are the time values of solution A adjusted to those of solution B by adding 61.50 minutes.

The Time Element is Apparent Only in the Initial Velocity.

After the initial stage of the hydrolysis has been passed, the velocity of hydrolysis gradually decreases and becomes the same irrespective of whether the cane sugar solution contained invert sugar or not at the beginning of the reaction. This is shown by the following experiments.

Two solutions were prepared exactly like solutions A and B described above. This time, solution A was allowed to react for 21 minutes before any readings were made, in order to permit any initial effect to disappear or become constant. Samples then were removed and examined in the polariscope. The changes in rotation together with the corresponding times calculated from the beginning of the hydrolysis are given under A in Table III. By following the hydrolysis of solution B in the same way, it was found that when the latter had been undergoing hydrolysis for 93.99 minutes its composition was the same as that of A when the latter had been undergoing hydrolysis for 30.9 minutes. By placing this point of solution B (93.99 minutes and 2.320° rotation) on the curve for solution A, in Figure II, at the point where A has the same rotation (2.320°), we find that this corresponds in time to 30.9 minutes for solution A. By subtracting the difference between these two times, 93.99 — 30.9 or 63.09 minutes from the time values of solution B (time values of B adjusted to those of A are given in the parenthesis under B in Table III) it is possible to plot the two curves together as in Figure II.

TABLE III.

Temperature 25°. Hydrogen ion conc. $10^{-4.4}$.

Solution A (like A in Table II)		Solution B (like B in Table II)	
Time	Rotation	Time	Rotation
21.02	2.823°	93.99 (30.90)	2.320°
24.01	2.670	96.99 (33.90)	2.177
28.00	2.463	101.00 (37.91)	1.994
32.02	2.265	105.01 (41.92)	1.807
36.02	2.074	109.03 (45.94)	1.632

It will be observed that over that portion of the time where the two hydrolysis curves overlap that they appear to be strictly superimposable and that, by inspection at least; the two curves on either side of the common portion still form a continuous curve. This indicates that the two solutions are now hydrolyzing with the same speed, and that the time element, or difference in velocity only occurs when one solution is starting and the other has been in progress for some time as indicated in Figure II.

The conclusion drawn from the data given in Table III and Figure II is supported also by some unpublished results from experiments carried out in this laboratory some years ago by Dr. F. M. Beegle. Beegle permitted a 5% cane sugar solution (B) to hydrolyze completely with invertase, then added 5 grms. more of finely powdered (so that solution would take

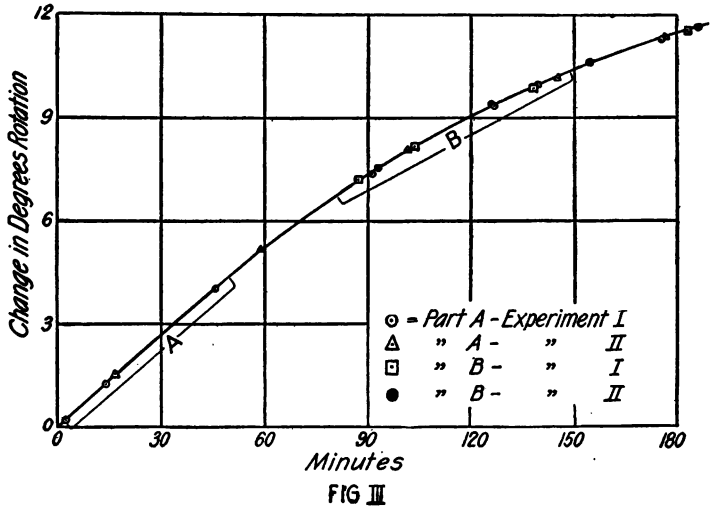

FIG III

place as rapidly as possible) cane sugar per 100 cc. of the solution and allowed the hydrolysis to continue. The velocity hydrolysis of the added portion of cane sugar was then compared with the velocity of a 10% cane sugar solution (A) after the latter had passed the 50% hydrolyzed stage. The change in rotation of the solutions and the corresponding times are given in Table IV, and the corresponding graphs in Figure III.

The calculated rotation of the 10% cane sugar solution A when 50% hydrolyzed was 3.80°. The rotations given in Table IV were determined by adding 5 cc. of a sodium carbonate solution to 20 cc. of the sugar solution and then examining it in the polariscope with a 200 mm. tube. The invertase correction for the rotations in this set of experiments was negligible. By subtracting 3.80° from the initial rotation of solution A, 10.64°, 6.84° is obtained as the change in rotation corresponding to the 50% hydrolysis stage. Locating this point, 6.84°, on the curve in Figure III and reading the corresponding time co-ordinate, the latter was found to be 82.0 minutes. The 5% hydrolyzed cane sugar solutions to which 5% more cane sugar had been added (that is solutions B I and B II in Table IV) had, in the case of B I a rotation 3.44° after the reaction had been in progress for 4.0 minutes. Solution A had the rotation 3.44° after it had been undergoing hydrolysis for 87.5 minutes or in other words it took solution A 87.5—82.0 or 5.5 minutes to undergo the same extent of hydrolysis as solution B I had undergone in 4.0 minutes. Similarly it was found that solution B II required only 9.5 minutes to undergo the same degree of hydrolysis as solution A underwent in 11.3 minutes. This again shows, as was demonstrated by the data given in Table II that the initial velocity, in the case of solution B, is greater than the velocity in such a solution, as A, where the reaction has been in progress for some time. But the data in Table IV also shows, as can be seen in Figure III, that the hydrolysis curve of solution B is super-imposable upon that of solution A and that the time-element is only apparent during the initial stage of a hydrolysis of cane sugar by invertase.

Besides the experiments of Table IV, another set was examined in which 2.5 grams of cane sugar per 100 cc. solution in-

stead of 10 grams were used and the velocity of hydrolysis beyond the 50% stage was compared with that of a solution containing 1.25 grams of cane sugar and 1.25 grams of inverted sugar per 100 cc. solution. These curves were found to coincide also.

TABLE IV.

Part A. 10 grms. cane sugar per 100 cc. Temp. 25°. Hydrogen ion conc. $= 10^{-4.26}$ (200 mm. tube).

I.		II.	
Time in minutes	Rotation	Time	Rotation
0.0	10.62°	0.0	10.64°
2.5	10.44	17.0	9.10
14.0	9.35	59.0	5.45
46.0	6.60	101.5	2.58
91.5	3.27	144.5	0.46
126.5	1.26	176.0	—0.70
175.5	—0.66	222.0	—1.81
∞	—3.06	260.0	—2.20
		∞	—3.04

Part B. 5 grms. cane sugar and 5 grms. inverted cane sugar per 100 cc. Temp., Invertase conc. and hydrogen ion conc. same as in A.

I.		II.	
Time	Rotation	Time	Rotation
0.0 (83.5)	—	0.0 (83.5)	—
4.0 (87.5)	3.44°	9.5 (93.0)	3.08°
20.5 (104.0)	2.46	42.5 (126.0)	1.25
55.0 (138.5)	0.75	71.0 (154.5)	0.00
100.0 (183.5)	—0.89	102.5 (186.0)	—1.00
146.0 (229.5)	—1.90	132.5 (216.0)	—1.68
219.0 (302.5)	—2.60	164.0 (247.5)	—2.17
∞ —	—3.09	225.5 (309.0)	—2.65

Does Invertase Action Involve Two or More Consecutive Reactions?

It will be observed upon inspection of the curves, in Figure I, part C, for the 1% cane sugar solutions containing 3% and

4% added glucose that they are slightly convex to the time axis at the start, or in other words, the initial velocity of hydrolysis under these conditions seems to increase. This increasing effect is so small, however, and the measurements involve the determination of such small absolute changes in the extent of the hydrolysis that it might be attributed to possible experimental errors. It was decided therefore to repeat these determinations in somewhat different manner. Instead of using the polariscope which is accurate in this case to 1 milligram of hydrolyzed cane sugar in 50 cc., a copper reduction method (for details see the experimental part of the paper) which has a higher degree of precision, 0.1 milligram of hydrolyzed cane sugar per 50 cc. of solution, was adopted.

In order to avoid the large amount of cuprous oxide that would be formed if the copper method were used in the case of the above 1% cane sugar solution containing 3% or 4% of added glucose, a 0.5% cane sugar solution, containing no added glucose, was used. This solution had about the same speed of hydrolysis as the 1% solution under the above conditions. A hydrolysis of a 1% cane sugar solution without any added glucose was followed also by means of the copper method for the sake of comparison, Part C, Table V. In Table V are given the results obtained and these results are plotted also in Figure IV.

Dr. Vosburgh, of this laboratory, has shown (his results are to be published shortly) that certain invertase preparations when acidified with hydrochloric acid to adjust the hydrogen ion concentration of the solution, do not always give reproducible results. For this reason, the hydrogen ion concentration of the 0.5% cane sugar solution was adjusted in two ways. The results given in Part A of Table V were obtained by using hydrochloric acid, and those in Part B by using an sodium acetate-acetic acid buffer mixture. In this way, if the velocities obtained in the two cases were the same then any abnormality in the velocity of hydrolysis that might appear cannot be attributed to the method of adjusting the hydrogen ion concentration of the solutions.

TABLE V.

Part A. 0.5% cane sugar solution. 2 cc Invertase per 100 cc. solution. Temperature 25°. Hydrogen ion, $10^{-4.4}$ moles per liter and adjusted by means of HCl.

Time	Mg. Cu.	Mg. cane sugar hydrolyzed per 50 cc.
1.03	11.28	4.02
2.14	22.95	9.30
3.01	30.34	13.44
4.03	40.37	18.14
5.03	49.10	22.22
6.03	57.52	26.20
7.00	65.46	30.09
8.00	74.15	34.26
9.04	82.25	38.23
10.01	90.18	42.19
11.02	98.16	46.20

Part B. Same solution and conditions except sodium acetate—acetic acid used for adjusting the hydrogen ion concentration.

Time	Mg. Cu.	Mg. cane sugar hydrolyzed per 50 cc.
1.03	12.62	4.30
2.02	22.27	8.90
3.04	31.56	13.43
4.03	40.56	17.90
5.03	48.95	22.02
6.03	56.59	25.60
7.00	64.25	29.35

Part C. 1% cane sugar solution, other conditions same as for B.

Time	Mg. Cu.	Mg. cane sugar hydrolyzed per 50 cc.
0.52	13.78	3.22
1.06	20.20	6.59
1.56	27.17	9.82
2.08	33.87	13.20
2.65	41.19	16.75
3.23	49.37	20.67
3.88	58.86	25.72
4.41	65.57	28.86

From the data in Table V and the shape of the curves in Figure IV, it is evident again that this increase in the initial velocity

of hydrolysis, at least in the case of the 0.5% cane sugar solution, shows up just as in the case of the curves Part C, Figure I.

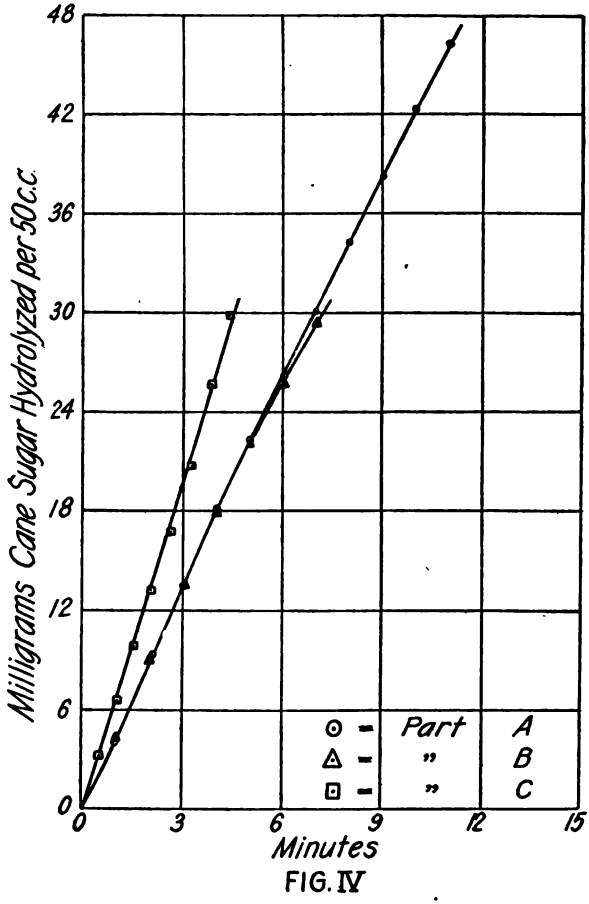

FIG. IV

To gather more evidence still on this point, the results obtained by other investigators from dilute sugar solutions were examined and plotted in the same way. Here again this same increase of the initial velocity was found to occur. Thus experiments 31 A and 31 B of Nelson and Vosburgh[6] in which a 0.4% cane sugar solution was used and similar experiments—7, p. 337, and F, p. 341—described by Michaelis and Menten (loc. cit.) in which 0.178% and 0.821% cane sugar solutions

respectively were used, all showed this increase in the initial velocity.

The Retarding Influence of Very Small Amounts of Glucose on the Velocity of Hydrolysis.

TABLE VI.

Conc. cane sugar = 1 g. per 100 cc. Conc. Invertase=0.5 cc. per 100 cc.
Conc. Hydrogen ion = $10^{-4.4}$. Temperature = 25°.

Conc. of Glucose %	Init. Rotation	Rotation after 50 min.	Change X 100
0.0	358.83°	358.25°	58
0.5	359.95	359.43	52
1.0	1.08	0.60	48
2.0	3.34	2.92	42
3.0	5.60	5.23	37
4.0	7.87	7.52	35

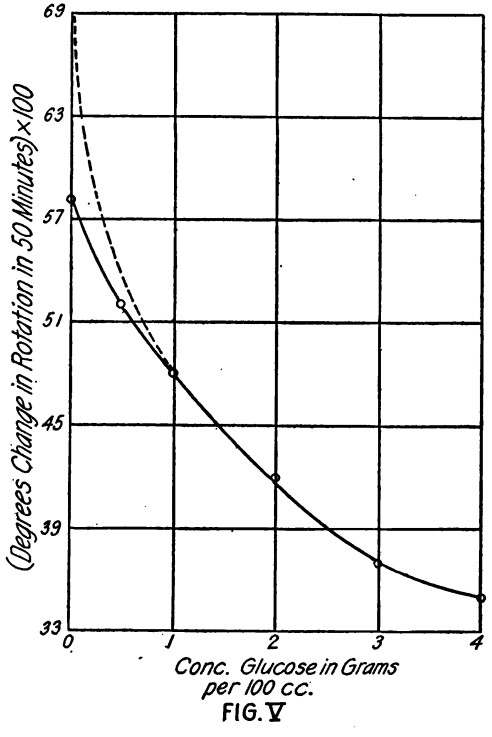

FIG. V

In Table VI is recorded the data obtained in studying the retarding influence of varying amounts of added glucose upon the rate of hydrolysis of a 1% cane sugar solution. It will be observed, especially by the inspection of curves in Figure V, a graph of the data, that, although the retardation is increased by augmenting the glucose concentration, yet each successive increment of the retardant has less and less effect. Thus, the measured effect of 0.5 grams of glucose at the beginning of the reaction is 58 — 52 or 6 units (see last column in Table VI) while when 3 grams were added then a decrease of 21 units was observed. In the first instance, the specific retardation (the specific retardation is defined as the influence of each unit of 1 gram of glucose under the conditions of the experiment) was $\frac{58-52}{0.5}$ or 12 and in the last $\frac{58-34}{3}$ or 7. Furthermore it must be borne in mind that at the time, 50 minutes after the start of the reaction*, when the readings were made, there was present in the solution containing no glucose about 0.17 grams per 100 cc. of invert sugar formed in the hydrolysis that had taken place up to this time. If the retarding influence of the invert sugar is considered as practically the same as that of glucose, then the specific effect of the 0.17 grams of invert sugar should be greater than that of 0.5 grams of glucose, since the retarding effect increases with decreasing amounts of the monoses present. If this is true, the value obtained for the "initial velocity of hydrolysis" must be too low due to the greater specific retardation of small amounts. This merely points out that the measured retardation effects of the small amounts must be too low and that in the light of this the true curve representing the retardation should rise very rapidly as it approaches the velocity axis and may possibly be asymptotic to it. As a consequence, the probable character of the true retardation curve is indicated by the dotted line in Figure V.

* It will be readily seen that, if there be no time effect at the start of the reaction, the change in rotation after 50 minutes will be on the initial linear portion of the hydrolysis curve with this concentration of enzyme. This would be a consequence of the results of Nelson and Vosburgh[6], who have shown that the velocity of the reaction is proportional to the invertase concentration.

The retarding effect of the invert sugar, as it is formed, therefore should cause a hydrolysis curve of a cane sugar solution to fall off rapidly from the start. This is, however, not the case, as has been shown above. A tentative explanation therefore suggests itself, that some other phenomenon besides retardation occurs which tends to offset the latter, thus giving rise to the constant or slightly increasing initial velocities obtained in this work.

EXPERIMENTAL

Preparation of Invertase. The invertase was prepared from yeast which had been allowed to autolyze. The filtered solution had been kept in a stoppered bottle for about three years previous to its purification. In general, the procedure followed was that described by Nelson and Born[7]. It was found that treatment of the autolyzed and filtered yeast solution with ammonia (stirring to prevent local alkalinity) until it was just short of being neutral to litmus, was very helpful in removing the precipitate obtained by the lead acetate. The invertase was not precipitated again after dialysis but was treated with toluene, bottled and kept in an ice-box. No loss in activity was noticed throughout the period of the investigation.

Cane Sugar. The cane sugar used was a good commercial grade such as Domino or Jack Frost which had been recrystallized from alcohol according to the method of Cohen and Commelin[8] and dried in a 0.04 mm. vacuum at 50° over sulphuric acid.

Glucose and Fructose. The glucose was a very pure commercial grade, obtained from the Corn Products Co., N. Y., and was recrystallized from acetic acid according to the method of Hudson and Dale[9]. It was washed with alcohol and dried at 50° over sulphuric acid at 0.04 mm. In order to remove the last traces of acetic acid, the drying was repeated over stick caustic soda.

The fructose was twice recrystallized from acetic acid according to Vosburgh[10]. It was noticed that, if the alcohol washings were combined with the filtrate and if the mixture

were placed in the ice-box, a large quantity of fructose could be recovered after about a week's standing. The fructose was dried in the same manner as the glucose, except that the temperature was maintained at 30°. To prevent decomposition and absorption of moisture, the sugars were kept in the dark in stoppered bottles inside a dessicator. They all exhibited the correct rotations.

The invert sugar used in this work was made up from equal quantities of glucose and fructose.

Preparation of Solutions, Table I. The method, with a few modifications, described by Nelson and Vosburgh[6] was used for the experiments given in Table I. 200 cc. of a sugar solution, of twice the concentration required, were added to an equal volume of invertase solution, also of twice the required concentration. A stream of air was blown through the solution to insure rapid and thorough mixing. Both the cane sugar and invertase solutions had been kept in the thermostat for at least 2 hours before mixing to permit them to acquire the correct temperature.

Solutions, Table II, III and V. The cane sugar solutions in this case, were made up by the aid of specific gravity tables[11] and corrected for 25° according to Schönrock's formula[11]. The required amount of powdered cane sugar was placed in a nonsol bottle. Water and the required amount of buffer were added so that on the addition of a definite volume of stock invertase solution (18.05 cc. or some other selected quantity) the final volume would be exactly 900 cc. Vigorous agitation by a current of air was resorted to.

Solutions, Table VII. The same procedure was followed as described for solutions, Table I, save that 25 cc. portions of sugar and invertase solutions were used instead of 200 cc.

Hydrogen ion adjustment. All the experiments were carried out at a hydrogen ion concentration of $10^{-4.4}$ moles per liter. For experiments described in Tables I and V, Part A, 0.01 molar hydrochloric acid was used according to the method of Nelson and Vosburgh[6] (that is—comparing the solutions

colorimetrically with citrate solutions that had been standardized by the electrometric method). In the electrometric standardization of the citrate solutions, a saturated potassium chloride-calomel half cell and a saturated potassium chloride bridge were used (Fales and Mudge[12]). In all the other solutions the hydrogen ion concentration was adjusted by means of sodium acetate-acetic acid buffer according to Michaelis[13]. This latter method was checked and found satisfactory.

Measurements of Volume and Time. The measuring flasks, burettes and pipettes were carefully calibrated. In the experiments described in Tables II, III, and V, the pipettes used had a time delivery from 8 to 12 seconds in order to eliminate any error in ascertaining the time of stopping the reaction when taken as a mean. The 18 cc. invertase pipette delivered in 7.5 seconds. They were all held vertically and removed when the free flow ceased. It was found that an operator can duplicate results by this method with a precision of better than $\pm$ 0.01 cc. In the other experiments ordinary calibrated pipettes were employed.

The time of delivery of the pipettes and the length of time of the reactions (that is—the time from the introduction of the enzyme to the interruption of the hydrolysis) were followed with a stop watch which could be read to 0.2 of a second and therefore the time intervals in all the Tables, except I, are precise to at least 0.01 minute.

All weights are so-called "weights in air."

Stopping the reaction. The reaction was stopped by running the 50 cc. samples from the reaction bottle in the thermostat, into 5 cc. of 0.2 molar sodium carbonate. The procedure was reversed in the experiments of Table VII. In those of Table I this operation was done in 60 cc. flasks and then made up to volume.

Determination of Extent of Hydrolysis. In all the experiments save those recorded in Table V, the degree of hydrolysis was determined in a 400 mm. jacketed tube which had been calibrated. The temperature was maintained at 25.00° $\pm$ 0.05°

by pumping water through the tube from the thermostat. The hydrolyses were all conducted at 25.043° ± 0.005°.

A Schmidt and Haensch triple field instrument at a half shadow angle of 2° was used in conjunction with a 1,500 candle power quartz mercury vapor lamp. The light was filtered through an Eastman No. 74 Wratten filter which had been tested with a Lummer-Brodhun spectrophotometer. This test showed that practically none of the mercury lines was transmitted except 546 $\mu\mu$ Readings could be made after some practice with a precision better than 0.01°.

Determination of Initial Angle. In obtaining the initial angles, Table I, 25 cc. of water and 25 cc. of cane sugar solution of double strength were run into 5 cc. of sodium carbonate solution, and this made up to 60 cc. and read in the polariscope. A correction was then made for the rotation that the invertase would have if present. In the other experiments, instead of the 25 cc. of water, 25 cc. of a double strength invertase solution were employed so as to conform to the rest of the readings in the same experiments.

Copper Method. The method of Thomas and Quisumbing of this laboratory, which is to be published soon, was used for determining the degree of hydrolysis in Table V.

Beakers, containing 50 cc. samples, 5 cc. 0.2 M sodium carbonate and 25 cc. each of the modified Fehling's solutions A and B, were immersed in a water bath kept at 80° ± 1°. for 30 minutes. The cuprous oxide was filtered off, washed and dissolved in dilute nitric acid. A small amount of sulphuric acid was added to this solution which was evaporated to dryness on a hot plate. The copper was determined finally by the thiosulphate method.

To construct the calibration curves for "reducing sugar", the following procedure was employed. Standard cane sugar and invert sugar solutions were made up. Definite amounts of these were added to 5 cc. of sodium carbonate into which the correct number of cc. of invertase solution had been run previously. Water was then added so that the final volume would be 55 cc. The analysis was then performed as described above. The addition of the invertase was necessary as it formed a cop-

per compound and thus tended to make the results too high unless allowed for. As the amount of this copper compound varied with the concentrations of the other substances present, it could not be corrected for and was therefore included in the calibration mixture. These calibration curves were constructed separately for each hydrolysis in order to eliminate any error due to varying composition of the Fehling's solutions. The data for these curves are given in Table VIII.

TABLE VIII.

For PartA, Table V		For Part B, Table V		For Part C, Table V	
Mg. Cane Sugar hydrol. per 50 cc.	Mg. of Copper	Mg. Cane Sugar hydrol. per 50 cc.	Mg. of Copper	Mg. Cane Sugar hydrol. per 50 cc.	Mg. of Copper
0.00	2.61	0.00	2.73	0.00	6.02
2.00	5.29	2.00	6.51	2.00	11.09
4.00	11.80	4.00	11.93	4.00	15.47
6.00	16.45	6.00	16.34	6.00	19.08
8.00	20.73	8.00	20.49	8.40	23.77
10.00	23.98	10.00	24.63	9.60	26.75
20.00	44.33	15.00	35.68	15.00	37.46
30.00	65.25	20.00	44.87	20.00	48.11
40.00	85.85	25.00	55.40	25.00	57.40
50.00	105.62	30.00	65.67	30.00	68.01
		35.00	75.05		

The points on the curves were so chosen that they corresponded to every two milligrams of cane sugar hydrolyzed up to the first 10 milligrams and to every five or ten milligrams of hydrolyzed cane sugar thereafter.

SUMMARY

(1) The initial velocity of the hydrolysis of cane sugar in the presence of invertase was found generally to be constant for a considerable period after the beginning of the reaction.

(2) A sucrose solution, containing added invert sugar, hydrolyses with a different initial velocity than that manifested during the reaction by a partially hydrolyzed cane sugar solution of the same composition.

(3) This difference in influence upon the velocity of the hydrolysis, between invert sugar added to the reaction at its be-

ginning and that formed during the hydrolysis, renders the method of Michaelis and Menten for determining the dissociation constants of the so-called sugar-invertase compounds valueless.

(4) It was found that the initial velocity of hydrolysis of dilute sucrose solutions or solutions to which a considerable amount of glucose had been added appeared to increase for a short period after the beginning of the reaction.

(5) The specific retardation due to glucose decreases as the concentration of hexose is increased and finally reaches a minimum. This indicates that the true initial velocity may be very great and (when the facts in section 4 are considered) that the hydrolysis probably consists of a series of consecutive reactions.

BIBLIOGRAPHY

[1] Henri—Lois générales de l'action des diastases. Paris (1903).

[2] Brown—*J. Chem. Soc.*, **81**, 373, (1902).

[3] Hudson—*J. Am. Chem. Soc.*, **30**, 1160, 1564, (1908).

[4] Armstrong—*Proc. Roy. Soc.*, **73**, 516, (1904).

[5] Michaelis and Menten—*Biochem. Zeit.*, **49**, 333, (1913).

[6] Nelson and Vosburgh—*J. Am. Chem. Soc.*, **39**, 797, (1917).

[7] Nelson and Born—*J. Am. Chem. Soc.*, **36**, 393, (1914).

[8] Cohen and Commelin—*Z. physik. Chem.*, **64**, 29, (1908).

[9] Hudson and Dale—*J. Am. Chem. Soc.*, **39**, 790, (1917).

[10] Vosburgh—*J. Am. Chem. Soc.*, **42**, 1696, (1920).

[11] Browne—*Handbook of Sugar Analysis*, (1st edition), page 30 and appendix, page 1.

[12] Fales and Mudge—*J. Am. Chem. Soc.*, **42**, 2434, (1920).

[12] Michaelis—*Die Wasserstoffionenkonzentration*, page 184.

[13] Colin and Chaudun—*Compt. Rend.*, **167**, 338, (1918).

VITA

Harold Lester Simons was born in New York City on July 15, 1897. He graduated from the College of the City of New York in June, 1917, receiving the degree of Bachelor of Arts. He entered the Graduate School of Pure Science of Columbia University in July, 1917, and received therefrom the degree of Master of Arts in June, 1918. He was Laboratory Assistant in Chemistry at that institution from February, 1918 to February, 1919. He was co-author (with Dr. H. L. Fisher) of a paper on "Dimethyl Tartronate" published in the *Journal of the American Chemical Society*, **43**, 628, (1921).